*BACTERIOPHAGE-SPECIFIC PROTEINS IN E. COLI INFECTED WITH AN RNA BACTERIOPHAGE**

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Communicated by W. Barry Wood, Jr., October 28, 1966

The f2 group of RNA coliphages (f2, MS2, R17, fr), first isolated by Loeb and Zinder,[1] are closely related, small viruses with single-stranded RNA of about one million molecular weight (for a review of RNA phages, see ref. 2). The number of proteins encoded in the viral RNA is therefore small. To date, two phage-specified proteins have been identified chemically or enzymatically in infected cells, the phage coat subunit and an RNA synthetase,[3–5] and the presence of a third protein has been inferred from genetic experiments.[6, 7] By analysis of radioactive proteins present in phage-infected cells, Haywood and Sinsheimer have provided evidence for the presence of four phage-specific, noncoat proteins in cells infected with coliphage MS2.[8]

We have recently described a method for labeling phage-specific proteins in *E. coli* infected with MS2 under conditions where host protein synthesis is virtually eliminated by actinomycin.[9] Such cells synthesize predominantly phage coat protein, the bulk of which is made later in the infectious cycle than is noncoat protein. The identification of noncoat protein in those studies depended on the incorporation of radioactive histidine, which is not present in the virus coat. In this report we describe the fractionation of phage-specific proteins by polyacrylamide gel electrophoresis. The results show that in addition to phage coat, three discrete proteins are present in infected cells. One of these proteins is missing in cells infected with an amber mutant with a defect in phage maturation. In confirmation of our earlier results,[9] it was found that coat protein constitutes about 80 per cent of the total phage-specific protein synthesized in the infected cell. In addition, preliminary findings are presented on the identification of noncoat proteins made in cell extracts under the direction of MS2 RNA and on the presence of minor proteins in the phage particle itself.

Materials and Methods.—The source of most of the bacterial strains and of bacteriophage MS2 has been given previously.[9, 10] Bacteriophage f2 and an amber mutant of f2 (sus-11) were kindly supplied by N. D. Zinder, as were *E. coli* K38, a nonpermissive host, and *E. coli* K37, an amber-suppressing strain.[11]

The procedures for sensitization of *E. coli* C3000 to actinomycin and for incorporation of amino acids into protein after infection with MS2 have been described.[9]

To prepare the radioactive protein for electrophoresis, the labeled cells were centrifuged at 10,000 *g* for 5 min, washed once with cold saline containing nonlabeled amino acids, and the cell pellet was resuspended in a small volume of water. The cells were broken by sonication for 3 min and all labeled protein was solubilized by addition of $^1/_{10}$ vol of glacial acetic acid, freshly deionized urea to a concentration of 0.5 *M*, sodium dodecyl sulfate (SDS) to a concentration of 1.0%, and mercaptoethanol to a concentration of 0.01 *M*.[12] After incubation at 37° for 60 min, the solution was centrifuged at 30,000 *g* for 10 min, and the supernate, which contained all the radioactive protein, was passed through coarse G25 Sephadex equilibrated with 20% sucrose, 0.1% SDS, and mercaptoethanol 0.01 *M*. For electrophoresis of the protein effluent, concentrated buffer was added to give the appropriate concentration (see below).

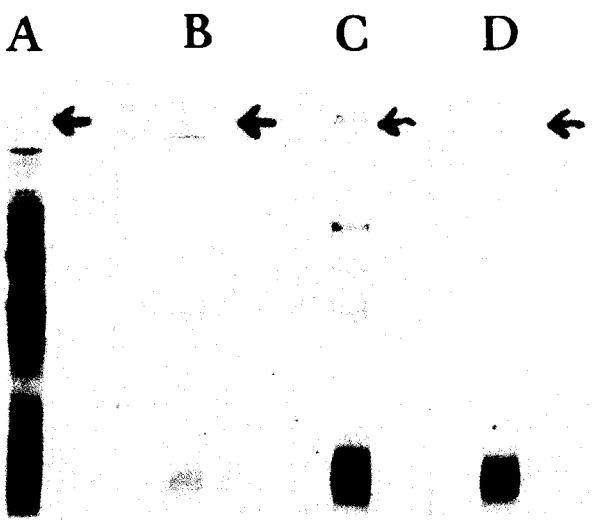

FIG. 1.—Radioautogram of dried gels, prepared as described in the text. (*A*) C^{14}-proteins prepared from growing *E. coli* in the absence of actinomycin. A 0.2-ml sample containing 50,000 cpm, derived from 0.1 ml of the original culture, was applied to the gel. (*B*) C^{14}-proteins prepared from actinomycin-treated, uninfected cells; 1000 cpm, derived from 0.3 ml of culture, was applied. (*C*) C^{14}-proteins prepared from actinomycin-treated cells infected with MS2 at a multiplicity of 20; 11,000 cpm, derived from 0.3 ml of culture, was applied. (*D*) C^{14}-amino acid-labeled phage particles, grown in actinomycin-treated cells and purified as indicated in the text; 11,000 cpm was applied. Electrophoresis was carried out at pH 7.2, and the dry gels were exposed to X-ray film for 11 days. The origin is indicated by the arrows.

Polyacrylamide gel electrophoresis was carried out at 25° at two different pH's: pH 8.9, using the procedure described by Davis,[13] or pH 7.2, using the procedure described by Summers *et al.*[12] In all cases 0.1% SDS and 0.5 *M* urea were in the gels, and 0.1% SDS in the buffers. Electrophoresis at pH 8.9 was done in 10-cm-length 7½% polyacrylamide gels at 5 ma per tube and run until the tracking dye (bromphenol blue) had moved 9 cm (about 3 hr). At pH 7.2, 10% polyacrylamide gels were used. They were 6 cm in length and were run at 5 ma per tube for 4 hr, at which time the tracking dye had moved about 4 cm.

Radioactive proteins in the gel were detected by two procedures: radioautography of dried slices of gel, as described by Fairbanks, Levinthal, and Reeder,[14] or by slicing the gel into 1-mm segments, eluting with 0.1% SDS, and counting the eluted protein in a liquid scintillation counter. With the latter procedure, about 90% of the applied radioactivity was recovered. The radioautograms were traced with a Joyce-Loebl recording microdensitometer.

Protein synthesis in cell extracts was carried out as previously described,[15] except that the S30 fraction was prepared from *E. coli* strain Q13, kindly supplied by W. Gilbert. The product was prepared for electrophoresis as described for the infected cell extract, except that acetic acid was omitted, EDTA (pH 7.6) was present at a concentration of 0.05 *M*, and mercaptoethanol at a concentration of 0.05 *M*.

The procedure followed for the purification of MS2 has been described.[10]

Chemicals were obtained from sources noted previously.[9, 10]

Results.—Number of phage-specific proteins made in infected cells: In the first experiment to be described, actinomycin-treated bacteria were infected with MS2 in the presence of C^{14}-lysine, C^{14}-arginine, C^{14}-isoleucine, and C^{14}-valine, and at the end of 50 min. the proteins were solubilized and prepared for electrophoresis as decribed in *Methods*. (By 50 min after infection, the synthesis of noncoat proteins has ceased and the amount of coat protein present is nearly maximal.[9]) A second

portion of the culture which had not been infected, and a third portion which had not been treated with actinomycin nor infected, were prepared for electrophoresis in the same way. Electrophoresis of each sample was performed at pH 8.9 and at pH 7.2 as described in *Methods*. In addition, samples of MS2, grown in actinomycin-treated cells in the presence of the C^{14}-amino acid mixture and purified in CsCl as previously described,[10] were also solubilized and electrophorized. At the end of the electrophoretic run, the gels were sliced, dried, and applied to X-ray film. The results are presented in Figures 1, 2, and 3.

Figure 1 shows a typical radioautogram of the dried gels, and Figures 2 and 3 are microdensitometer tracings of the radioautograms. As shown in the figures, normally growing *E. coli* showed a variety of labeled proteins, whereas actinomycin-treated *E. coli* showed a specific pattern of protein synthesis, i.e., the persistent synthesis of a small number of proteins. (A similar observation was reported by Haywood and Sinsheimer.[8]) In actinomycin-treated, infected cells, four new protein peaks appear (Figs. 1, 2*C*, and 3*C*), one of which is present in great excess and corresponds to phage coat protein. As seen in the figures, one of the noncoat proteins is present in small amount, and this component appears to be somewhat variable in electrophoretic mobility in different preparations. As shown by the

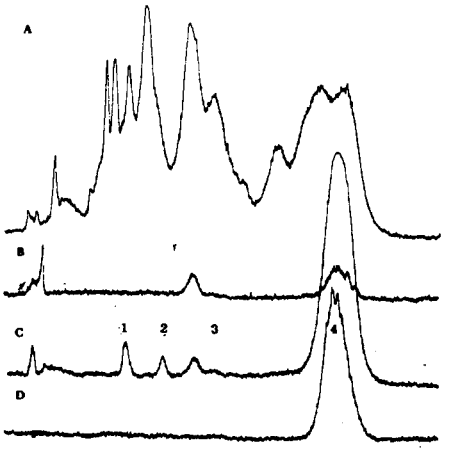

FIG. 2.—Densitometer tracings of the radioautograms shown in Fig. 1. *A*, *B*, *C*, *D* are the same as noted in Fig. 1. The origin is indicated by the vertical line or first peak on the left. Phage-specific proteins are numbered *1–4*. The 3rd component is more evident in Figs. 4 and 6.

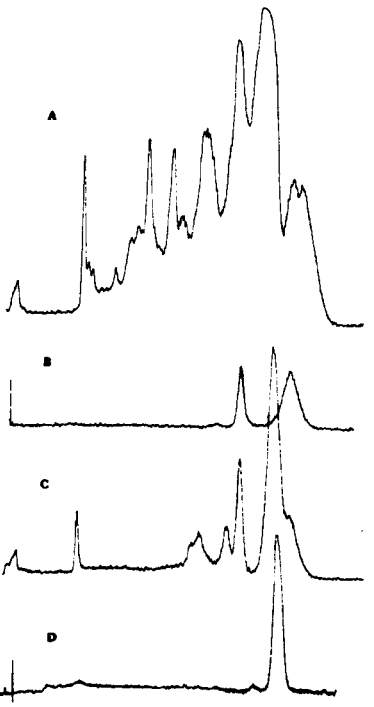

FIG. 3.—Densitometer tracings of radioautograms of C^{14}-proteins electrophorized at pH 8.9. (*A*) Normal *E. coli*. (*B*) Actinomycin-treated *E. coli*. (*C*) Actinomycin-treated *E. coli* infected with MS2. (*D*) Purified MS2 grown in actinomycin-treated cells. Origin is indicated by vertical line or first peak on the left.

results of other electrophoretic runs, the amount of radioactive material at or near the origin is quite variable and often entirely absent (Fig. 4). It is presumably an aggregate of radioactive protein and not a separate protein component.

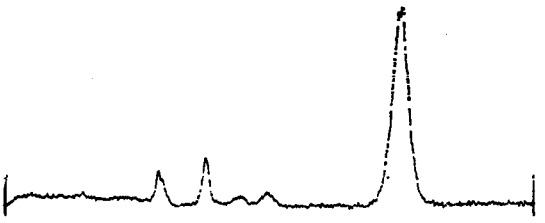

FIG. 4.—Densitometer tracing of protein from actinomycin-treated, infected cells, showing no radioactive material at the origin. The sample was prepared as described in *Methods* and electrophorized at pH 7.2. The origin is at the left.

That no other phage protein is present in the coat fraction is shown by the electrophoretic pattern of C^{14}-histidine-labeled proteins, isolated from actinomycin-treated, infected cells as described above. As shown in Figure 5, phage-specific proteins labeled with histidine, an amino acid lacking in MS2 coat protein, are present in two major peaks and a third minor (and variable) peak, but not in the coat protein region. (The control peaks can be identified from Figs. 2 and 3.) These results also establish that the first and second protein peaks are not aggregated coat protein.

In order to detect additional phage-specific proteins which might be present in very small amounts, gel slices were exposed to film for longer periods. After

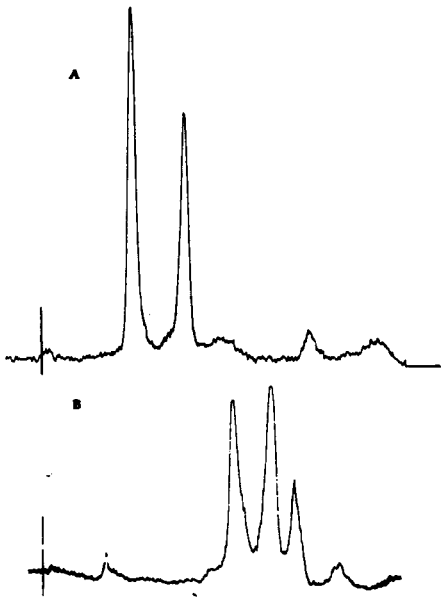

FIG. 5.—Densitometer tracings of C^{14}-histidine-labeled protein prepared from actinomycin-treated, infected cells. (*A*) Electrophoresis at pH 7.2; 5000 cpm was applied and the film exposed for 12 days. (*B*) Electrophoresis at pH 8.9; 7000 cpm was applied and the film exposed for 5 days. The origin is at the left.

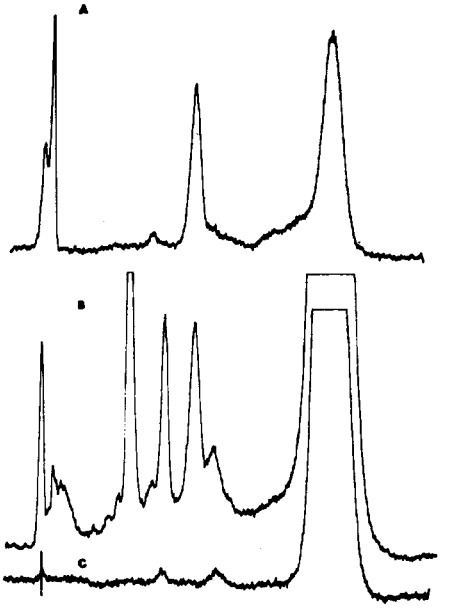

FIG. 6.—Densitometer tracings of radioautograms developed after long exposure to dried gels. The same gels used for the radioautogram and tracings shown in Figs. 1 and 2 were exposed to X-ray film for 33 days, and the film was traced. (*A*) Actinomycin-treated *E. coli*. (*B*) Actinomycin-treated *E. coli* infected with MS2. (*C*) Purified MS2 grown in actinomycin-treated cells.

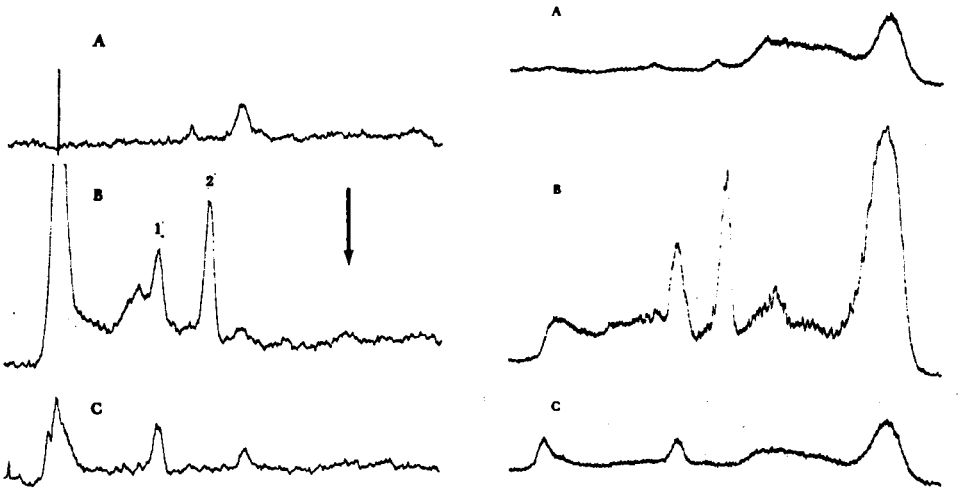

FIG. 7.—Analysis of proteins from cells infected with sus-11 or its revertant. The figure on the left shows the proteins labeled with C^{14}-histidine; that on the right shows the proteins labeled with C^{14}-arginine, C^{14}-lysine, C^{14}-isoleucine, and C^{14}-valine. (*A*) Actinomycin-treated cells. (*B*) Actinomycin-treated cells infected with the sus-11 revertant. (*C*) Actinomycin-treated cells infected with sus-11. The origin corresponds with the first peak at the left of each tracing, and the arrow indicates the position of coat protein in a companion gel.

exposure for 33 days, the same gels used for Figures 1 and 2 yielded the tracings presented in Figure 6. As shown in Figure 6*A*, a new trace component is seen in uninfected cells, but no new phage-specific proteins are apparent (Fig. 6*B*). An interesting observation emerges, however, in the tracing of the protein from purified phage particles (Fig. 6*C*). As seen in Figure 6*C*, two minor peaks are present, one of which corresponds to phage-specific peak 2 and the other to phage-specific peak 3. Neither peak 1 protein nor the protein seen in uninfected cells is detectable in the phage.

Absence of a specific protein in an amber mutant of f2: One way of identifying the proteins separated by electrophoresis is to examine the phage-specific proteins formed in nonpermissive cells infected with amber mutants of the RNA phage, since the defective gene gives rise to incomplete polypeptide chains.[16] For this purpose, actinomycin-treated *E. coli* C3000, a nonpermissive strain, was infected with sus-11, an amber mutant of f2 which has nonsense mutations in both the coat protein cistron and in the cistron which codes for a protein required for the protection or organization of viral RNA.[17] The labeled amino acid in one experiment with this mutant was histidine, so that the mutation in the coat protein could be ignored, and in a second experiment a mixture of C^{14}-amino acids was used. For comparison a companion batch of cells was infected with a revertant of sus-11 which was selected on the nonpermissive host, *E. coli* K38. A third batch of cells was not infected. The amounts of C^{14}-histidine incorporated into total protein (hot TCA-insoluble radioactivity[9]) after 50 minutes were as follows: uninfected cells, 150 cpm per ml of culture; sus-11 infected cells, 400 cpm; sus-11 revertant infected cells, 1200 cpm. The results of electrophoresis of these preparations are shown in Figure 7. As shown in the figure, cells infected with sus-11 lack the second protein

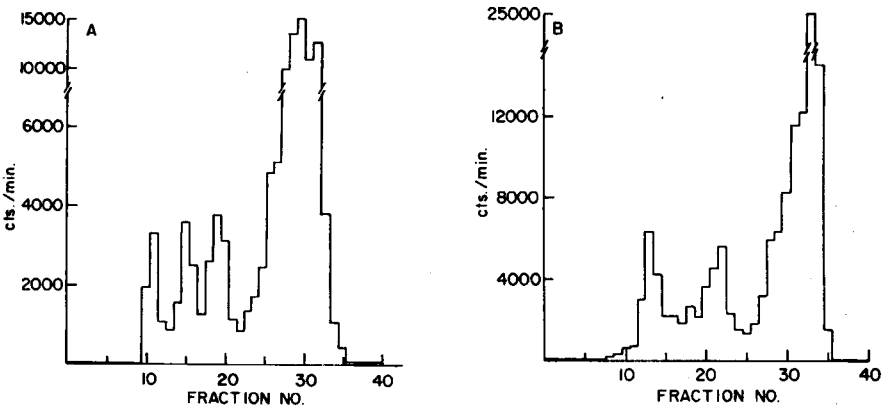

FIG. 8.—Electropherogram of proteins made in cell extracts with MS2 RNA as messenger. Gels were sliced as described in *Methods*, and protein was eluted with 0.1% sodium dodecyl sulfate and counted. The first segment includes the origin. (*A*) Proteins made in infected cells, labeled with C^{14}-amino acid mixture. (*B*) Proteins made in cell extracts, labeled with C^{14}-amino acid mixture.

component. This result establishes that the first and second proteins are not aggregates of the same subunit, and identifies peak 2 as the "RNA-protecting protein." Similar studies are now in progress with a series of amber and temperature-sensitive mutants of MS2 to identify the other protein peaks.

Electrophoretic analysis of proteins synthesized in E. coli extracts with MS2 RNA as messenger: Bacteriophage RNA has been shown to direct the synthesis in *E. coli* extracts of intact phage coat as well as histidine-containing, noncoat proteins.[15, 18, 19] The lack of purified reference proteins of the latter type has precluded more precise identification of these proteins. By applying the same techniques employed in the analysis of phage-specific proteins formed in infected cells, we have identified three principal protein products in the cell-free system (Fig. 8). In a separate experiment, the first and second peaks have been shown to contain histidine and are therefore not coat protein aggregates. In double-label experiments, in which C^{14}-cell-free product was mixed with H^3-infected cells and the mixture prepared for electrophoresis, the first peak seen in Figure 8*B* corresponded in mobility to peak 1 from infected cells, and the third peak seen in figure 8*B* corresponded to coat protein. However, the middle peak of the cell-free product did not coelectrophorese with any of the proteins seen in infected cells, although it was similar in mobility to the variable peak 3. We presume that this peak represents altered RNA-protecting protein. As shown in Table 1, protein closely related to coat protein in electrophoretic mobility constituted about 70 per cent of the total protein product, and each of the other components was about 15 per cent. These relative amounts are similar to those found with phage-specific proteins made in infected cells (Table 1). More detailed studies of the proteins made in cell extracts are in progress in order to establish their relationship to the proteins found in infected cells.

Discussion.—Three cistrons have been identified in the f2 group of RNA coliphages by complementation tests:[20, 21] one for the coat protein subunit, one for an RNA-synthesizing enzyme, and a third for an "RNA-protecting protein." In the present study, four phage-specific proteins have been detected in infected cells.

TABLE 1

PER CENT OF TOTAL COUNTS IN EACH PEAK

Source of proteins	Total Counts (%) 1st Peak	2nd Peak	Coat protein
Infected cells (*a*)	8	11	81
(*b*)	10	12	78
Cell extract (*a*)	13	16	71
(*b*)	14	17	69

These results were obtained from the experiment described in the legend of Fig. 8. (*a*) and (*b*) are duplicate electrophoretic runs.

One of these is coat protein, a second is the "RNA-protecting protein," and we infer that a third is the RNA synthetase. In addition to these known proteins, a fourth, variable protein component is found in infected cells. Whether the latter is the product of a fourth cistron, whose function is not vital for virus development, or is a breakdown product or aggregate of one of the other proteins is not as yet clear.

We have already commented on the striking excess of phage coat protein production in infected cells, compared with the other proteins.[9] Even at 50 minutes post-infection, the amount of coat protein is seven times that of any other protein component. Similarly, in cell extracts the amount of phage coat produced is also in great excess ($4^1/_2$-fold). Moreover, both in infected cells and in cell extracts the two reproducible minor protein peaks are about equal. These similarities suggest that the same regulatory mechanisms may be operating in each case. The availability of a simple procedure for quantitating the different phage proteins synthesized offers the opportunity of exploring these mechanisms both in the infected cell and in cell-free extracts.

The preliminary findings on the presence in phage particles of two minor phage-specific protein components suggest that the RNA phage is structually more complex than thought heretofore. The possibility that RNA coliphages contain a protein which protects the RNA from nuclease cleavage and perhaps serves as the site of attachment to cells is suggested by the properties of certain mutants of the phage,[6, 7] and by the effect of 5-fluorouracil in producing RNA-deficient nonabsorbing particles.[6, 22] A rough estimate of the amount of the minor components found in phage particles, one of which corresponds to the "RNA-protecting protein," indicates that a phage particle may have a single molecule of each. At this level, however, it is difficult to distinguish structural protein from contaminating protein, and other experiments are being done to settle this point satisfactorily. Also, it remains to be shown that the two minor components are actually different proteins, since one might be a breakdown product of the other.

A final comment concerns the selective inhibition of protein synthesis by actinomycin. As shown by the electropherograms of protein from actinomycin-treated, noninfected cells, only two major radioactive peaks and one trace component are detectable. Of these, the fastest-moving peak is probably not protein, since it is present in maximal amount in samples of cells taken immediately after addition of radioactive amino acids. This result therefore suggests that a specific *E. coli* protein or class of proteins is made on relatively stable messenger RNA.

Summary.—Four phage-specific proteins have been detected by polyacrylamide gel electrophoresis in lysates of actinomycin-treated *E. coli* infected with an RNA bacteriophage. One of the proteins, present in excess over any of the others, corresponded in mobility to the phage coat protein. A second component has been

identified as the "RNA-protecting protein" previously inferred from genetic studies, and a third protein is presumed to be the RNA synthetase. The fourth protein is variable in electrophoretic mobility and is present in much smaller amount than any of the others. The "RNA-protecting protein," as well as the minor variable protein, were also detected in normal phage particles. Fractionation of the proteins made in *E. coli* extracts in the presence of phage RNA as messenger showed two distinct minor proteins in addition to phage coat protein. The relative amounts of the minor proteins synthesized in cell extracts and in infected cells were similar. It is suggested that the same control mechanisms may operate in the infected cell and in cell-free extracts regulating the translation of polycistronic viral RNA.

Note added in proof: We have recently learned that E. Viñuela, I. Algranati, and S. Ochoa (personal communication) have also used polyacrylamide gel electrophoresis to fractionate the phage-specific proteins made in actinomycin-treated cells infected with MS2.

* This research has been supported by a grant from the National Institutes of Health, U.S. Public Health Service. M. P. O. is a postdoctoral fellow, and K. E. is a predoctoral trainee of the National Institutes of Health.

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